

*Final Technical Report for NAG 2-1005*  
*(Part 1 of 2). P.I. = Gary W. Conrad*  
*(27 pp + 5 Figs)*

Effects of Microgravity on Embryonic Quail Eye Development

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Running Heading: Quail Eye Development in Microgravity

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## ABSTRACT

Immunohistochemical methods were used to stain neurofilament protein in corneal nerves of Embryonic Day 16 (E16) quail eyes that had been fixed in 4% paraformaldehyde at room temperature for several months. Fixation was according to the methods used by the Mir 21/NASA 2 Avian Developmental Biology Flight Experiments for quail embryos incubated on the Mir Space Station. After fixation, corneas were pretreated to improve immunohistochemical visualization of neurofilaments. A sequential combination of three pretreatments [microwave heating in saline G, followed by extraction with sodium dodecyl sulfate (SDS) at 37°C, followed by digestion with hyaluronidase at 37°C], produced increased antibody staining of corneal nerve neurofilament proteins, compared with corneas subjected to no prior pretreatments. Darker nerve staining and increased numbers of fine branches were observed, together with lower background staining after such pretreatments. In contrast, use of any single pretreatment or pair of pretreatments resulted in only slight and inconsistent enhancement of nerve staining. Only the sequential combination of all three pretreatments resulted in consistently better nerve staining.

**KEY WORDS:** antibodies, antigen unmasking, anti-neurofilament, immunohistochemistry

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(Part 2 of 2). P.I. = Gary W. Conrad  
(21 pp + 2 Figs)

1

Increased Neurofilament Immunostaining of Corneal Nerves in Long-Term Paraformaldehyde-Fixed Embryonic Quail Eyes After Microwaving, Sodium Dodecyl Sulfate Extraction, and Hyaluronidase Digestion

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Running header: Antigen Retrieval in Formalin-fixed Corneas

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## INTRODUCTION

One of the objectives of the current international space program is to determine if embryonic animals and plants develop normally in microgravity. As part of the joint Mir 21/NASA 2 Avian Developmental Biology Flight Experiments, we sought to determine what effects microgravity would have on eye development, particularly innervation of the corneas of quail embryos incubated aboard the Russian Space Station, Mir. Since corneal innervation is dense, as visualized in whole mount preparations (1), that tissue provides an excellent system for studying the effects of microgravity on innervation.

Fertilized eggs of Japanese quail (*Coturnix coturnix japonica*) were collected on Earth and transported at subincubation temperatures on U.S. Space Shuttle vehicles to Mir. Incubation was initiated on the space station, and continued until the desired day of development prior to hatching. The embryos were fixed in 4% paraformaldehyde while still inside their shells. They remained in the fixative solution at room temperature for several months aboard Mir until they were returned to Earth by another Shuttle flight.

Conventionally, formaldehyde or paraformaldehyde fixation of nerve tissue is performed for a short period, such as a few minutes or hours, and often at 4°C. Long fixation times at room temperature, such as those necessitated by space flight schedules, were expected to affect proteins, lipids and carbohydrates. In aldehyde-fixed tissue, proteins undergo crosslinking (6), thus also immobilizing proteoglycans, together with their glycosaminoglycan side chains (8, 10). Such cross-linked extracellular matrices create barriers to antibody diffusion. In addition, the fixation process can interfere with antigen recognition because the addition of formaldehyde groups masks antigenic sites, thereby further reducing the effectiveness of immunohistochemical staining (4, 5, 19, 31). Neurofilament epitopes, in particular, have been shown to become masked, after as little as 24 hours of formaldehyde fixation (14).

Methods for permeabilizing such long-term fixed tissue have been sought to improve antibody penetration, and facilitate epitope unmasking: e.g., low pH, high pH, high salt

concentrations, high temperatures and proteolytic enzyme digestion of the tissue (4, 14, 17, 19). Pressure cooking (23) and microwave oven irradiation also have been shown to increase antigen accessibility in formalin-fixed, paraffin-embedded tissue (3, 27, 29).

The focus of this investigation was to devise a technique that could be used to enhance visualization of nerves in whole-mount preparations of flight and control corneas that had been stored in 4% paraformaldehyde at room temperature from two to six months. Microwave pretreatment was included because of its demonstrated efficacy in antigen unmasking (4, 24, 28, 29). SDS was included because of its ability to solubilize non-crosslinked proteins (25), thereby facilitating diffusion of reagents into cells. Hyaluronidase was included because its enzymic cleavage of several, widely distributed, classes of glycosaminoglycans (2, 13, 18), facilitates diffusion of reagents through extracellular matrices. Following three separate pretreatments, which were used a) individually, b) sequentially in pairs, or c) as a sequence of all three pretreatments, immunohistochemical procedures were used to visualize neurofilament proteins in corneal nerves.

## METHODS

### *Incubation and Eye Fixation*

All experiments were conducted according to the National Research Council's animal use guide (22). Fertilized Japanese quail eggs (*Coturnix coturnix japonica*), obtained from Oak Ridge Game Farm, Maysville, AR, were stored at 17°C for an average of 7 days, then incubated for 16 days at  $37^{\circ} \pm 1.0^{\circ}\text{C}$ . Embryonic Day 16 (E16) embryos were removed from their shells, and the spinal cords were severed at the base of the skull. Non-punctured eyes were removed from the orbit, freed of eyelid tissue, immediately placed into paraformaldehyde (Sigma, St. Louis, MO) solution (4% (w/v) in 0.2 M Na/K-PO<sub>4</sub> buffer, pH 7.2, prepared according to NASA Protocol) (21), and then stored at room temperature (20-22°C) in the same solution for two to six months, with occasional swirling for agitation.

### *Dissection of Corneas*

Eyeballs were transferred from fixative to a solution of saline G for a ten-minute rinse with agitation; thereafter, the tissue remained in fresh saline G for dissection. The anterior half of each eye was removed with fine iris scissors. The lens, retina and iris were removed, and most scleral tissue was trimmed away, leaving the cornea surrounded by only a narrow scleral margin. Corneas were then cut in half to allow unimpeded access of antibodies into the interior of the stromal extracellular matrix, where most of the linear regions of corneal nerves are located (1). Trimmed corneas were then transferred immediately into individual vials of saline G and maintained on ice until used (one to two hours).

### *Pretreatment of Corneas Prior to Immunohistochemical Staining Procedures*

Fixed corneas were pretreated with one, or with a sequence of two or three methods. The pretreatments were always performed in the following order: first, microwaving; second,

incubation in SDS; third, incubation in hyaluronidase. Control corneas were incubated in their respective buffers, but without SDS or hyaluronidase; in these cases, for clarity, the word *buffer* is written in italics.

*Microwave heating, sodium docetyl sulfate extraction, and hyaluronidase digestion*

All microwaving was done prior to the SDS or hyaluronidase incubations. Each cornea to be microwaved was transferred to a separate 50 ml plastic centrifuge tube containing 10 ml saline G at room temperature. Each tube containing a cornea was loosely capped and placed into a glass beaker which was then covered with clear plastic wrap. The beaker and tube containing the cornea were then microwaved (Whirlpool, Model MT6120XYB-2, 800 Watts, Benton Harbor, MI) on full power for 25 sec. During microwaving, the saline reached temperatures of 88-92°C. After such treatment, the tissue was immediately returned to its original vial of room temperature saline G. Control corneas not microwaved remained at room temperature in saline G during the procedure.

All SDS incubations were performed after the microwaving step, and prior to hyaluronidase incubation. Experimental tissue was incubated with continuous rocking motion for one hour at 37°C in 1.0% (w/v) SDS (ICN Biomedicals, Aurora, OH) in phosphate-buffered saline (PBS), pH 7.4 (6). SDS *buffer* control tissues also were similarly incubated at 37°C, but in PBS alone. Experimental and control corneas were then rinsed three times (60 min each) in saline G + 0.2% (v/v) Triton X-100 (Packard, Downers Grove, IL) at 37°C with agitation.

Experimental tissue was incubated with continuous motion overnight at 37°C in type IV-S bovine testicular hyaluronidase (Sigma) [880 units/mg; 0.1 mg/ml in 0.1 M sodium phosphate buffer, (pH 5.3) containing 0.15 M NaCl] (30). Hyaluronidase *buffer* control tissues also were incubated at 37°C, but in buffer lacking enzyme. Experimental and control corneas then were rinsed in saline G + 0.2% Triton X-100 twice (10 min each) at room temperature.

### *Immunohistochemistry*

All steps below were performed at room temperature unless stated otherwise. To inactivate endogenous peroxidases, control and experimental corneas were rinsed in saline G + 0.2% Triton X-100 + 0.3% (v/v) hydrogen peroxide ( $H_2O_2$ ) (Sigma) three times (20 min each), followed by one 15 min rinse in saline G + 0.2% Triton X-100. After one, two, or three of the procedures, tissues were rinsed four times (15 min each) in blocking solution, consisting of saline G + 0.2% Triton X-100, 5% (w/v) powdered milk (Carnation Natural Nonfat Dry Milk, Nestle Food Co., Glendale, CA), 1% (w/v) Bovine Serum Albumin (BSA) (Sigma), and 1% (v/v) normal filtered goat serum (Hazelton Research Products, Denver, PA). Corneas then were incubated in the primary antibody [Anti-Neurofilament 200 kDa immunoglobulin G, developed in rabbits (Sigma #N-4142), diluted 1:1500 in the blocking solution] at 0-4°C overnight with constant movement, followed by rinsing in blocking solution (four 15 min rinses). Corneas then were incubated in the secondary antibody [Goat anti-rabbit IgG peroxidase conjugate (Sigma #A-0545), diluted 1:200 in the blocking solution] for 3 hrs at room temperature with constant movement, followed by one 15 min rinse in blocking solution and four 15 min rinses in saline G + 0.2% Triton X-100. Tissues were then pre-incubated in the dark at room temperature in a solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma #D-5905) (0.25 mg/ml in saline G + 0.2% Triton X 100) with constant movement. Corneas then were incubated at room temperature in Metal-enhanced DAB (Pierce kit #34065, Rockford, IL, prepared according to kit instructions) for 5-15 min with constant movement. Continual monitoring of tissue color development was necessary to prevent overdevelopment. Following DAB visualization, corneas were rinsed two times (15 min each) in saline G + 0.2% Triton X-100 and were stored at room temperature in 100% glycerol. After at least 24 hours of storage in glycerol, corneas were photographed with a Leica MPS 52 camera (Heerbrugg, Switzerland) mounted on a Wild M5 stereo dissecting



microscope (Heerbrugg, Switzerland). Nerve staining for each cornea was evaluated individually by two people, then a rating score was determined for each cornea. Ratings were from 0 to 4, with 0 indicating little staining, with no or few nerves visible, and uneven background color, and 4 indicating strong nerve staining and uniform background color.

#### *Other pretreatments tested*

Although trypsinization of formaldehyde-fixed tissue has been reported to moderately increase availability of neurofilament epitopes (14), in the present study we found that trypsin pretreatment (Type III from bovine pancreas, Sigma T-8253, activity 10,000-13,000 BAEE units/mg protein, 2.0 mg/ml CMF saline G + 0.2% Triton X-100, pH 7.4, 1 hr with agitation, at 37°C) of long-term paraformaldehyde-fixed corneas did not result in increased neurofilament staining in corneal nerves (data not shown). A variety of other pretreatments of such corneas also did not enhance neurofilament staining, including: papain (0.6 mg/ml CMF saline G + 0.2% Triton X-100, pH 5.3, 1 hr with agitation, at 65°C); pronase (4.0 mg/ml CMF saline G + 0.2% Triton X-100, pH 7.9, 1 hr with agitation, at 50°C); chondroitinase ABC (0.15 units/ml 0.1 M Tris-acetate buffer, pH 7.3, overnight with agitation, at 37°C); keratanase (0.5 units/ml 0.2 M sodium acetate buffer, pH 7.4, overnight with agitation, at 37°C). Similarly, neurofilament staining in such fixed corneas was not enhanced by a variety of mechanical pretreatments, including: freeze-thaw cycles, sonication, or perforation of the epithelium and/or endothelium into the stroma.

## RESULTS

Table 1 summarizes the effects of the various pretreatment procedures on the degree of immunohistochemical staining of corneal nerves (patterns shown photographically in Figures 1 and 2). Controls: Control corneas that had been fixed in 4% paraformaldehyde, but then were neither microwave pretreated nor incubated in SDS *buffer* or hyaluronidase *buffer* before immunohistochemical staining, showed only very faint nerves (Figure 1a). Other control corneas, not microwaved, but incubated sequentially only in SDS *buffer* and hyaluronidase *buffer* solutions, showed slight nerve staining with very little detectable branching (Figure 1b). The background was spotted and uneven in color. Use of single treatment methods prior to neurofilament antibody staining resulted in somewhat improved nerve staining (Figures 1c-e): Microwave: Corneas that were microwaved, then incubated in SDS *buffer*, followed by hyaluronidase *buffer* incubation, showed moderately well-stained nerves, with more nerve branching detected than in control corneas (Figure 1c). In addition, the background of such microwaved corneas was more uniform in color compared with controls. SDS: Corneas incubated in SDS, followed by incubation in hyaluronidase *buffer*, showed moderately increased nerve staining (Figure 1d). This improvement was detected in about half the corneas, particularly in corneas cut in two before immunostaining. Hyaluronidase: Corneas pretreated with SDS *buffer* then incubated in hyaluronidase enzyme showed moderate nerve staining in most trials (Figure 1e). Some fine nerve branches were visualized, and the background was a uniform, light color.

Figure 2 shows corneas that were pretreated with two or three different methods sequentially prior to the immunostaining step. Microwave + hyaluronidase: After microwave treatment, followed by incubation in SDS *buffer*, and then incubation in hyaluronidase enzyme, nerve branches were moderately well-stained, and the background was of uniform tan color (Figure 2a). Microwave + SDS: Microwave treatment, followed by SDS incubation, and then by incubation in hyaluronidase *buffer*, resulted in poor nerve staining and an uneven spotted background (Figure 2b). SDS + Hyaluronidase: SDS incubation, followed by incubation in

hyaluronidase enzyme (no prior microwave treatment), resulted in poorly stained nerve branches and a spotty background (Figure 2c).

Microwave + SDS + Hyaluronidase: Consistently, the best results in corneal nerve staining were obtained in corneas subjected sequentially to all three pretreatments: microwaving, followed by incubation in SDS, followed by incubation in hyaluronidase enzyme (Figure 2d). Nerves were darkly stained, with many fine branches, and the background had an even, light tan color.

## DISCUSSION

Results reported here indicate, first, that in heavily-fixed corneas in the absence of any pretreatments, very little corneal nerve staining occurs; second, that when fixed corneas are pretreated with microwaving, or SDS, or hyaluronidase (singly or in pairs of pretreatments), corneal nerve staining is improved somewhat; third, that when fixed corneas are pretreated with all three conditions, nerves are consistently well stained against an even, light background.

It is uncertain by what mechanisms these procedures work in unmasking antigenic sites in formaldehyde-fixed tissues. Morgan et al. (20) suggested that calcium ions may form complexes with proteins during formaldehyde fixation, resulting in the masking of certain antigens. Precipitation of those ions might result in antigen unmasking, and heat might be needed for their release from the complex. In addition, heating of fixed tissue would be expected to cause some protein denaturation and limited protein hydrolysis. Antigenic sites might then be rendered accessible in the slightly altered proteins (23). Higher microwave heating temperatures for short time periods tend to result in better unmasking than do lower temperatures for longer periods: e.g., microwave heating of fixed tissues at 90°C for 10 minutes allowed better immunostaining than did heating at 60°C for 120 minutes (12).

SDS is an anionic detergent that solubilizes lipids and denatures proteins, often enhancing digestion of released molecules and extracted tissues by a variety of enzymes (25). For this investigation, we used SDS to enhance the accessibility of hyaluronidase to its glycosaminoglycan substrates.

Hyaluronidase catalyzes cleavage of glycosidic bonds of hyaluronic acid, chondroitin sulfates, and some non-epimerized glucuronic acid linkages of dermatan sulfate (13, 18). The latter two glycosaminoglycans, together with keratan sulfate (7), occur abundantly as the predominant proteoglycans of the vertebrate corneal stroma (9, 15, 16). It is not certain if aldehyde fixatives cross-link polysaccharide chains (6), although glycogen content is preserved and proteoglycans become well-fixed (10). Enzymic removal of such glycosaminoglycan side

chains of proteoglycans would be expected to increase the ability of antibodies to diffuse into the extracellular matrix in which the corneal nerves are found.

We used SDS *buffer* and hyaluronidase *buffer* solutions as controls for the detergent and enzyme treatment steps. Buffers, including dilute acetic acid in the absence of proteolytic enzymes, have been used to facilitate antigen retrieval and unmasking (2, 11, 17, 23). However, our results show that incubating corneas in the *buffer* solutions used for SDS or for hyaluronidase treatments did not lead to dramatically better staining than in corneas not treated with such *buffers*. In contrast, inclusion of the respective experimental agents (SDS and hyaluronidase enzyme) resulted in enhanced nerve staining compared with their corresponding *buffer* controls.

In the present study, moderate improvement in corneal nerve staining resulted when two pretreatment methods were used sequentially on a single sample. However, the most effective and consistent antigen unmasking occurred in corneas subjected to a triple pretreatment regimen of first microwaving, then SDS incubation, followed by hyaluronidase incubation (Figure 2d). In addition, the background of such pretreated corneas was usually more uniform in color than the background of corneas given no treatment (Figure 1a), those given a single treatment (Figures 1 c-e), or a combination of two treatment methods (Figures 2 a-c) prior to immunohistochemical staining.

## ACKNOWLEDGMENTS

We thank James McReynolds for comments on this manuscript. Research supported by NASA NAG 2-1005 and NAGW-2328, and NIH EY00952.

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Table 1 Intensity of corneal nerve immunohistochemical staining following various prior pretreatments of fixed tissue.

Cornea Figure Number	Pretreatment	Nerve Stain Rating
1a	none	0
1b	SDS <i>buffer</i> hyaluronidase <i>buffer</i>	0
1c	microwave SDS <i>buffer</i> hyaluronidase <i>buffer</i>	1
1d	SDS hyaluronidase <i>buffer</i>	1
1e	SDS <i>buffer</i> hyaluronidase enzyme	2
2a	microwave SDS <i>buffer</i> hyaluronidase enzyme	3
2b	microwave SDS hyaluronidase <i>buffer</i>	1
2c	SDS hyaluronidase enzyme	2
2d	microwave SDS hyaluronidase enzyme	4

Ratings:

0 = very little, or no, nerve staining; background staining faint or an unevenly distributed color

1 = nerves faintly visible

2 = moderately visible nerves; a few branches

3 = nerves containing some branches

4 = dark nerve stain with many fine branches; background color pale tan and uniformly distributed

Figure 1. E-16 quail half-corneas given individual pretreatments prior to neurofilament immunostaining.

- (a) Control cornea given no prior pretreatment procedures.
- (b) Control cornea given no microwave treatment, incubated in SDS *buffer* only, then incubated in hyaluronidase *buffer* only.
- (c) Microwave: Cornea microwaved in saline G, then incubated in SDS *buffer*, then in hyaluronidase *buffer*.
- (d) SDS: Cornea incubated in SDS, then in hyaluronidase *buffer*.
- (e) Hyaluronidase: Cornea incubated in SDS *buffer* then in hyaluronidase enzyme.

Bar: 1a, 1b = 0.5 mm; 1c-1e = 0.4 mm

Figure 2. E-16 quail half-corneas treated sequentially with two or three different pretreatments prior to neurofilament immunostaining.

- (a) Microwave + hyaluronidase: Cornea microwaved in saline G, incubated in SDS *buffer*, then incubated in hyaluronidase enzyme.
- (b) Microwave + SDS: Cornea microwaved in saline G, then incubated in SDS, followed by incubation in hyaluronidase *buffer*.
- (c) SDS + hyaluronidase: Cornea incubated in SDS, then incubated in hyaluronidase enzyme.
- (d) Microwave + SDS + hyaluronidase: Cornea microwaved in saline G, incubated in SDS, then incubated in hyaluronidase enzyme.

Bar: 2a-2d = 0.4 mm

Fig. 1

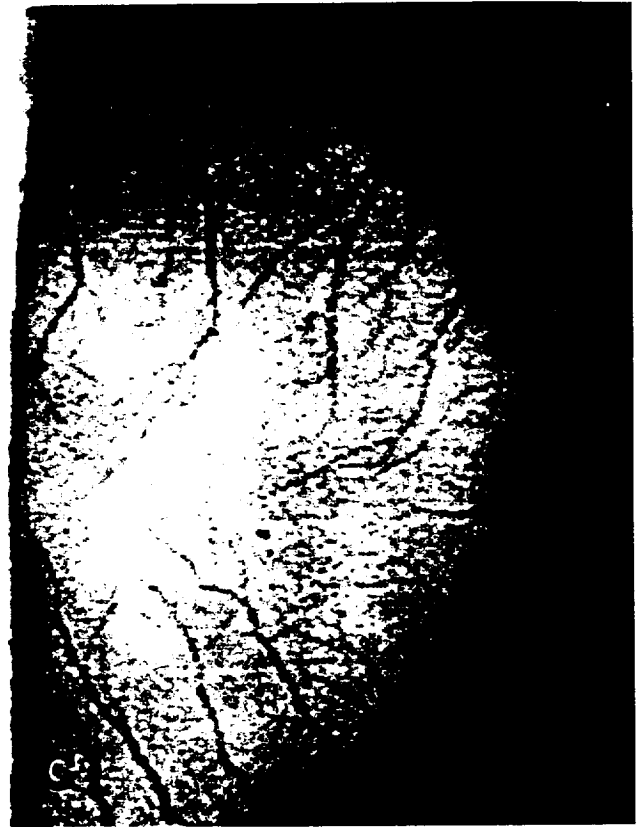


Fig. 2

